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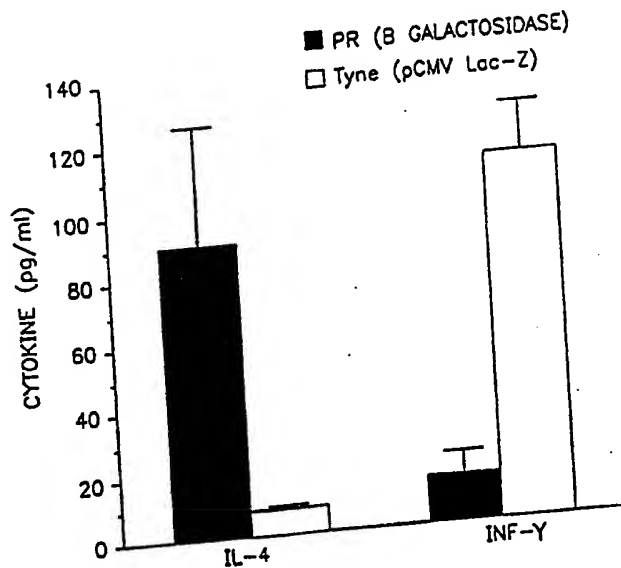
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(54) Title: METHOD FOR TREATING ALLERGIC LUNG DISEASE

(57) Abstract

The invention is directed to a method for treating both the early and late phases of allergic asthma by introducing naked polynucleotides which operatively encode for the asthma-initiating antigen into the host. The antigen-encoding polynucleotides are administered to host tissues which contain a high concentration of antigen presenting cells (e.g., skin and mucosa) relative to other host tissues. Expression of the asthma-initiating antigen encoding polynucleotides of the invention inside of antigen presenting cells (without substantial secretion therefrom) induces antigen tolerance while suppressing IgE antibody formation in the early phase of the disease, and also suppresses cytokine-mediated eosinophil accumulation in the late phase of the disease. Devices and compositions for use in the methods of the invention are also described.



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METHOD FOR TREATING ALLERGIC LUNG DISEASE

STATEMENT OF GOVERNMENT RIGHTS

- 5 This invention may have been made with Government support under Grant Nos. AR07567 and AR25443, awarded by the National Institutes of Health. The Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

- 10 The invention relates to a method for treating both the early and late phases of allergic lung disease. In particular, the invention relates to a method for immunizing a host against allergic asthma through use of asthma-initiating antigen-encoding polynucleotide compositions.

2. History of the Prior Art

- 15 Asthma is one of the common chronic lung diseases of industrialized countries. The airway narrowing which characterizes the disease is associated with antigen stimulated immune system activation, including elevation of antigen-specific IgE levels in the early phase of the disease and eosinophil infiltration of lung tissue in the late phase of the disease.
- 20 Specifically, during the early phase of the disease, activation of Th2 lymphocytes stimulates the production of IgE antibody, which in turn triggers the release of histamine and other immune mediators from mast cells. During the late phase of the disease, IL-4

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and IL-5 cytokine production by CD4⁺ helper T lymphocyte type 2 (Th2) cells is elevated. These cytokines are believed to play a significant role in recruiting eosinophils into lung tissue, where tissue damage and dysfunction result.

Persons suffering from allergic asthma are conventionally treated by immunization against the asthma-initiating antigen with an antigen-based composition. Antigen immunization limits the antigen-stimulated events of the early phase of allergic asthma, albeit at the risk of inducing IgE mediated anaphylaxis. However, such classical immunization schemes do not target the cytokine-mediated events of the late phase immune response in allergic asthma.

10 SUMMARY OF THE INVENTION

The invention consists of a method for treating allergic asthma in a host which reduces the allergic immune responses that are characteristic of both the early and late phases of the disease. This is accomplished according to the invention by administering an asthma-initiating antigen-encoding polynucleotide to the host in a manner which induces intracellular expression of the antigen in antigen presenting cells. The expressed antigen is presented to host CD4⁺ T lymphocytes in a manner which activates class 1 helper T (Th1) lymphocytes in preference to Th2 lymphocytes.

Thus, the polynucleotide immunization scheme of the invention allows the clinician to induce tolerance to an asthma-initiating antigen in a host with little risk of stimulating the Th2 lymphocyte mediated IgE antibody production and mast cell activation events which characterize the early phase of allergic asthma. In addition, the immunization scheme of the invention also reduces Th2 cell release of IL-4 and IL-5, thus substantially limiting the eosinophil accumulation in lung tissue which characterizes the late phase of allergic asthma. In this manner, the invention provides a more efficacious, less risk-intensive means of treating allergic asthma than is presently available in the art.

In practice, a suitable candidate for treatment according to the method of the invention is a host in whom allergic asthma has been diagnosed and for whom at least one asthma-initiating antigen (i.e., a proteinaceous antigen which triggers an allergic response in the host that results in asthmatic symptoms being experienced by the host) has been identified.

According to the method of the invention, the host is immunized with a pharmaceutical composition comprised of a recombinant expression vector (preferably a plasmid or cosmid, hereafter "polynucleotide composition"). The recombinant expression vector incorporates a polynucleotide which encodes the asthma-initiating antigen. To enhance Th1 lymphocyte activation to a therapeutically sufficient level, the polynucleotide composition is administered to a tissue of the host which contains a relatively high concentration of antigen presenting cells (e.g., skin or mucosa, such as the mucosa of the respiratory tract) as compared to other host tissues.

Advantageously, targeting the dense population of antigen presenting cells present in the skin and mucosa for expression of antigen permits relatively minute doses of polynucleotide composition to be applied toward a therapeutic effect in the host.

Where desired, the recombinant expression vector of the polynucleotide composition may also code for other therapeutically significant, biologically active peptides, such as immunostimulatory cytokines (e.g., TGF- β). Alternatively, such peptides or other therapeutically significant compounds may be administered in conjunction with the polynucleotide compositions of the invention.

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BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts the results of an ELISA for anti-NP IgG before intranasal introduction of naked pCMVRNP to Balb/c mice.

FIGURE 2 depicts the results of an ELISA for anti-NP IgG in an unanesthetized group of Balb/c mice.

FIGURE 3 depicts the results of an ELISA for anti-NP IgG in an anesthetized group of Balb/c mice.

FIGURE 4 is a photograph of the results of histological studies of skin at the point of entry for pCMVRNP in Balb/c mice showing uptake of the plasmid by mononuclear cells (APC's). An APC is indicated by an arrow; a tissue cell (not containing the plasmid) is indicated by a slashed line.

FIGURE 5 depicts the results of an ELISA for IgG 2A type antibodies in sera for mice (1) injected intradermally or intramuscularly with a polynucleotide encoding β -galactosidase, or (2) the enzyme by intradermal injection.

FIGURE 6 depicts the results of an ELISA for IgG 1 type antibodies in sera for mice (1) injected intradermally or intramuscularly with a polynucleotide encoding β -galactosidase, or (2) the enzyme by intradermal injection.

FIGURE 7 depicts the results of an ELISA for IgG 2A type antibodies in sera of the mice described with respect to FIGURE 5 after a booster injection of antigen.

FIGURE 8 depicts the results of an ELISA for IgG 1 type antibodies in sera of the mice described with respect to FIGURE 6 after a booster injection of antigen.

FIGURE 9 depicts the results of an ELISA for IgG 2A type antibodies in sera for mice (1) introduced by scratching the skin with tynes coated with a polynucleotide encoding β -galactosidase, or (2) the enzyme by intradermal injection.

FIGURE 10 depicts the results of an ELISA for IgG 1 type antibodies in sera for mice (1) introduced by scratching the skin with tynes coated with a polynucleotide encoding β -galactosidase, or (2) the enzyme by intradermal injection.

FIGURE 11 is a map of the pGREtk eukaryotic expression vector.

FIGURE 12 is a map of the pVDRtk eukaryotic expression vector.

FIGURE 13 depicts the results of an ELISA for total IgE antibody levels in mice after immunization with an antigen-encoding plasmid (pCMV-Lac-Z), the antigen itself (β galactosidase), or a control (non-encoding) plasmid (pCMV-BL).

FIGURE 14 depicts the results of an ELISA for antigen-specific IgE antibody levels in mice after immunization with an antigen-encoding plasmid (pCMV-Lac-Z), the antigen itself (β galactosidase), or a control (non-encoding) plasmid (pCMV-BL).

FIGURE 15 depicts the results of an ELISA for levels of IL-2 and INF γ after immunization of mice with an antigen-encoding plasmid (pCMV-Lac-Z) or the antigen itself (β galactosidase).

FIGURE 16 depicts the results of an assay to detect antigen-specific cell lysis by T lymphocytes from mice immunized by epidermal administration of pCMV-NP plasmid.

FIGURE 17 depicts the results of an assay to detect antigen-specific cell lysis by T lymphocytes from the mice described in FIGURE 16 in absence of pulsing of the cells with the antigen.

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FIGURE 18 depicts the results of an ELISA for anti- β -galactosidase antibodies after administration of (1) a polynucleotide encoding the enzyme by intramuscular or intradermal injection, and (2) the enzyme by intradermal injection.

FIGURE 19 depicts the results of an ELISA for anti- β -galactosidase antibodies in sera
5 from the mice described with respect to FIGURE 18 after a booster injection of antigen.

DETAILED DESCRIPTION OF THE INVENTION

Throughout this description, the preferred embodiment and examples shown should be considered as exemplars, rather than limitations on the invention.

I. DEFINITIONS

10 The following definitions are provided to simplify discussion of the invention. Those skilled in the art will, however, recognize that these definitions may be expanded to include equivalents without departing from the legitimate scope or spirit of the invention. For this reason, these definitions should not be construed as limiting the invention.

- 15 a. "Polynucleotide" refers to DNA or RNA and can include sense and antisense strands as appropriate to the goals of the therapy practiced according to the invention. Polynucleotide in this context may include oligonucleotides. Polynucleotides useful in the invention are those which are incorporated into recombinant expression vectors that include promoter and other sequences necessary for expression of the desired translation product(s); e.g., a peptide
20 or protein. The method of the invention can be practiced using known either viral or non-viral recombinant expression vectors, although the latter are preferred. Preferably, these vectors will incorporate complementary DNA (cDNA) which encode for the desired translation product(s).

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- b. "Polynucleotide composition" refers to a pharmaceutically safe polynucleotide free of a delivery vehicle (such as liposomes or colloidal particles); i.e., a "naked" polynucleotide in a carrier which will not impair antigen recognition of the polynucleotide.
- 5 c. "Asthma-initiating Antigen" refers to one or more proteinaceous antigens (1) to which the host has been determined to be allergic; and (2) which stimulate asthmatic symptoms in the host.
- 10 d. "Antigen Presenting Cells" or "APC's" include known APC's such as Langerhans cells, veiled cells of afferent lymphatics, dendritic cells and interdigitating cells of lymphoid organs. The definition also includes mononuclear cells such as (1) lymphocytes and macrophages which take up and express polynucleotides according to the invention in skin and (2) mononuclear cells (e.g., as depicted in histological photographs contained herein). These cells are not tissue cells but are likely to be antigen presenting cells. The most important of these with respect to the present invention are those APC's which are known to be present in high numbers in epithelia and thymus dependent areas of the lymphoid tissues, including epidermis and the squamous mucosal epithelia of the buccal mucosa, vagina, cervix and esophagus (areas with "relatively high" concentrations of APC's). In addition to their definitions set forth below, therefore, "skin" and "mucosa" as used
- 15 20 herein particularly refer to these sites of concentration of APC's.
- e. "Host" refers to the recipient of the therapy to be practiced according to the invention. The host may be any vertebrate, but will preferably be a mammal. If a mammal, the host will preferably be a human, but may also be a domestic livestock, laboratory subject or pet animal.
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- f. "Target tissue" refers to the tissue of the host in which expression of a polynucleotide is sought.
- g. "Skin" as used herein refers to the epidermal, dermal and subcutaneous tissues of a host.
- 5 h. "Mucosa" refers to mucosal tissues of a host wherever they may be located in the body including, but not limited to, respiratory passages (including bronchial passages, lung epithelia and nasal epithelia), genital passages (including vaginal, penile and anal mucosa), urinary passages (e.g., urethra, bladder), the mouth, eyes and vocal cords. Respiratory passages are the
10 primary target tissue for introduction of polynucleotide compositions to treat allergic asthma according to the invention.
- i. "Point of Entry" refers to the site of introduction of the naked polynucleotide into a host, including immediately adjacent tissue.
- 15 j. "Th1/Th2 Response(s)" refer, respectively, to types 1 and 2 helper T lymphocyte (Th) mediated immune responses. Th2 responses include the allergy-associated IgE antibody class as well as elevated levels of IL-4 and IL-5 cytokines by Th2 lymphocytes. Soluble protein antigens tend to stimulate relatively strong Th2 responses. In contrast, Th1 responses are induced by antigen binding to macrophages and dendritic cells. that is
20 induced preferentially by antigens that bind to and activate certain APC's; i.e., macrophages and dendritic cells. Th1 cells secrete IL-2, interferon (IFN)- γ and tumor necrosis factor (TNF)- β (the latter two of which are involved in macrophage activation and delayed-type hypersensitivity in response to antigen stimulation).

- k. "Synthesis" refers to well-known means of synthesizing polynucleotide sequences and may include isolation and purification of native polynucleotides.
- l. "Peptide" refers to small peptides, polypeptides, oligopeptides and proteins which have a desired biological effect *in vivo*.
- m. "Iontophoresis" refers to a known means of transdermal transmission presently used to deliver peptides continuously to a host. More specifically, it is a process that facilitates the transport of ionic species by the application of a physiologically acceptable electrical current. This process and other transdermal transmission means are described in Chien, *et al. Transdermal Drug Delivery*, "Novel Drug Delivery Systems", Ch. 7, part C, (Marcel Dekker, 1992), the relevant disclosures of which are incorporated herein by this reference for the purpose of illustrating the state of knowledge in the art concerning techniques for drug delivery.
- 15 n. "Detergents/Absorption Promoters" refers to chemical agents which are presently known in the art to facilitate absorption and transfection of certain small molecules, as well as peptides.
- 20 o. "Dermal" and "Epidermal Administration" mean routes of administration which apply the naked polynucleotide(s) to or through skin. Dermal routes include intradermal and subcutaneous injections as well as transdermal transmission. Epidermal routes include any means of irritating the outermost layers of skin sufficiently to provoke an immune response to the irritant. The irritant may be a mechanical or chemical (preferably topical) agent.

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- p. "Epithelial Administration" involves essentially the same method as chemical epidermal administration, except that the chemical irritant is applied to mucosal epithelium.
- q. "IL" refers to interleukin.
- 5 r. "IFN" refers to interferon.

II. DISCUSSION

A. Theory of the Invention

- The method of the invention exploits the unexpected discovery that asthma-initiating antigen-encoding polynucleotide compositions which are taken up and expressed in host
- 10 APCs (1) stimulate production of Th1 lymphocytes in preference to Th2 lymphocytes; (2) consequently suppress the IgE antibody production characteristic of the early phase of allergic asthma; and (3) consequently reduce the IL-4/IL-5 stimulated eosinophil infiltration of lung tissue characteristic of the late phase of the disease. Notably, administration of polynucleotide compositions which encode asthma-initiating antigens
- 15 (or fragments thereof) not only suppresses IgE antibody production, but also does so from the outset of therapy, thus reducing the risk of anaphylaxis posed by classical immunotherapy. Thus, the method of the invention effectively and immediately manipulates the T lymphocyte compartment of the host immune response to reduce both the IgE and cellular immune-mediated events associated with allergic asthma.
- 20 More specifically, the method of the invention introduces asthma-initiating antigens into the intracellular compartment of host APCs present in the target tissue, where the antigen is retained without substantial secretion therefrom (see, Examples IV through VII). This intracellular expression and retention of antigen preferentially stimulates Th1 responses against the antigen. Because the Th2 response to extracellular antigen achieved in

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classical immunotherapy is avoided, IgE production and IL-4/IL-5 release in response to extracellular antigen is also avoided.

For example, as shown in Examples VII and VIII, IgE and IL-4 levels in expressed antigen-challenged mice were surprisingly very low, while asthma-initiating antigen-specific CTL levels (Example IX) and Th1 cell secretion of $\text{INF}\gamma$ (Example VIII) were enhanced (as compared to protein challenged and control mice). The suppression of IgE and IL-4 production achieved in mice immunized according to the invention continued despite subsequent challenge with the plasmid or protein, even when combined with adjuvant (Examples VII-VIII). Thus, while both mice challenged with protein antigen and mice immunized according to the invention developed IgG mediated tolerance to the immunizing antigen, the latter mice suffered far less from the IgE mediated immune events which characterize the early phase of allergic asthma.

In addition, mice immunized according to the invention fare better in the late phase of allergic asthma than do protein-antigen immunized mice. In particular, the data in Example II demonstrate that administration of ovalbumin antigen-encoding polynucleotide compositions to murine models for allergic asthma produced (with adjuvant) up to a 90% reduction in eosinophil infiltration of lung tissue in the mice on subsequent asthma-initiating antigen challenge, as compared to control mice. Thus, the mice immunized according to the invention were protected from eosinophil infiltration of lung tissue far better than their protein-antigen immunized litter mates.

Thus, in contrast to classical allergic asthma immunotherapy, the asthma-initiating antigen-encoding gene immunotherapy of the invention abrogates both asthma-initiating antigen-specific and non-specific IgE production in the early phase of allergic asthma, protects the host from further production of IgE even on subsequent asthma-initiating antigen challenge, and reduces cellular lung infiltration and airway hypersensitivity in the late phase of the disease.

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B. Polynucleotide Compositions Useful in the Invention

1. Useful antigen-encoding polynucleotides and recombinant expression vector constructs

5 In allergic asthma, the symptoms of the disease are triggered by an allergic response in a host to an allergen. The polynucleotide sequences of many nucleic acids which code for asthma-initiating antigen allergens are known. All such polynucleotide sequences are useful in the method of the invention. Examples of some of the more common allergens for use in the invention are set forth below; those of ordinary skill in the art will be familiar with additional examples, the use of which is encompassed by the invention.

- 10 For use in the method of the invention, the recombinant expression vector component of the polynucleotide compositions of the invention may encode more than one asthma-initiating antigen, different peptides of an asthma-initiating antigen, or a combination of the two. The polynucleotides may encode for intact asthma-initiating antigen or T cell epitope(s) of an asthma-initiating antigen, engineered by means well-known in the art to
15 be non-secreting.

As noted above, many asthma-initiating antigen-encoding polynucleotides are known in the art; others can be identified using conventional techniques such as those described elsewhere below. Examples of known asthma-initiating antigen-encoding polynucleotides include cDNAs which code for IgE reactive major dust mite asthma-
20 initiating antigens *Der pI* and *Der pII* (see, Chua, et al., *J.Exp.Med.*, 167:175-182, 1988; and, Chua, et al., *Int.Arch.Allergy Appl. Immunol.*, 91:124-129, 1990), T cell epitope peptides of the *Der pII* asthma-initiating antigen (see, Joost van Neerven, et al., *J.Immunol.*, 151:2326-2335, 1993), the highly abundant Antigen E (*Amb aI*) ragweed
25 phospholipase A₂ (bee venom) asthma-initiating antigen and T cell epitopes therein (see, Dhillon, et al., *J.Allergy Clin.Immunol.*, 90:42-51, 1992), white birch pollen (*Ber vI*) (see,

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Breiteneder, *et al.*, *EMBO*, 8:1935-1938, 1989), and the *Fel d1* major domestic cat asthma-initiating antigen (*see*, Rogers, *et al.*, *Mol. Immunol.*, 30:559-568, 1993). The published sequences and methods for their isolation and synthesis described in these articles are incorporated herein by this reference to illustrate knowledge in the art
5 regarding asthma-initiating antigen-encoding polynucleotides.

In addition, expression (by the same or a different recombinant expression vector) or co-administration of therapeutically beneficial peptides such as TGF- β , TNF- β , IL-2 and IFN- γ enhance the Th1 response sought by the method of the invention. IL-2 and IFN- γ are of particular interest in this regard because, in recent clinical trials, IL-2 and gamma
10 interferon have proved toxic at dosages sufficient to interfere with production of IgE.

The polynucleotides to be used in the invention may be DNA or RNA, but will preferably be a complementary DNA (cDNA) sequence. The polynucleotide sequences used in the invention must be (a) expressible and (b) either non-replicating or engineered by means well known in the art so as not to replicate into the host genome. Illustrations
15 of the preparation of polynucleotides suitable for use in the invention follow. It will, however, be apparent to those skilled in the art that other known means of preparing nonreplicating polynucleotides may also be suitable.

In general, DNA sequences for use in producing therapeutic and/or immunogenic peptides of the invention can be obtained by several methods. For example, the DNA
20 can be isolated using hybridization procedures which are well known in the art. These include, but are not limited to: 1) hybridization of probes to genomic or cDNA libraries to detect shared nucleotide sequences; 2) antibody screening of expression libraries to detect shared structural features and 3) synthesis by the polymerase chain reaction (PCR). The development of specific DNA sequences encoding or fragments thereof, can
25 also be obtained by: 1) isolation of double-stranded DNA sequences from the genomic DNA; 2) chemical manufacture of a DNA sequence to provide the necessary codons for the polypeptide of interest; and 3) *in vitro* synthesis of a double-stranded DNA sequence

by reverse transcription of mRNA isolated from a eukaryotic donor cell. In the latter case, a double-stranded (cDNA) complement of mRNA.

5 A cDNA library believed to contain a polynucleotide of interest can be screened by injecting various mRNA derived from cDNAs into oocytes, allowing sufficient time for expression of the cDNA gene products to occur, and testing for the presence of the desired cDNA expression product, for example, by using antibody specific for a peptide encoded by the polynucleotide of interest or by using probes for the repeat motifs and a tissue expression pattern characteristic of a peptide encoded by the polynucleotide of interest. Alternatively, a cDNA library can be screened indirectly for expression of
10 therapeutic and/or immunogenic peptides having at least one epitope using antibodies specific for the peptides. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of cDNA of interest.

Screening procedures which rely on nucleic acid hybridization make it possible to isolate
15 any gene sequence from any organism, provided the appropriate probe is available. Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligopeptide stretches of amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must
20 be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA.

The polynucleotide which encodes each asthma-initiating antigen may be conjugated to
25 or used in association with other polynucleotides which code for regulatory proteins that control the expression of these polypeptides or may contain recognition, promoter and secretion sequences. Those of ordinary skill in the art will be able to select regulatory

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polynucleotides and incorporate them into the polynucleotide compositions of the invention (if not already present therein) without undue experimentation. For example, suitable promoters for use in murine or human systems and their use are described in *Current Protocols in Molecular Biology, supra* at Ch. 1.

- 5 Together with appropriate regulatory sequences, polynucleotide(s) for use in the invention are incorporated into a recombinant expression vector, preferably a non-viral plasmid or cosmid vector. Use of a non-viral vector, particularly one which comprises a replicator, will prolong expression of the gene in target tissues. Certain plasmid vectors are also good mediators of immune responses to immunogenic peptides because high
- 10 levels of expression are achieved when the gene encoding the peptides is incorporated into the vector.

The recombinant expression vectors (both viral and non-viral) most preferred for use in the invention are described in detail in co-pending, commonly assigned U.S. Patent Application Serial No. 08/593,554, the disclosure of which is incorporated herein by

15 reference for the purpose of illustrating vectors useful in the invention. Briefly, the preferred expression vectors for use in the invention include at least one palindromic, non-coding region (i.e., a region where the nucleotide sequence of one strand is the reverse complement of a corresponding region of the complementary strand) of at least 6 nucleotides in length. Each such palindromic region includes an unmethylated CG

20 dinucleotide sequence; i.e., at least two adjacent nucleotides, where one such nucleotide is a cytosine and the other such nucleotide is a guanine.

In double-stranded molecules, each CG dinucleotide sequence present in the palindromic region is itself palindromic; i.e., the cytosine of the CG sequence on one strand is paired with a guanine in a CG sequence on the complementary strand. In single-stranded

25 molecules, the relative position of each CG sequence in the palindromic dinucleotide is preferably 5'-CG'3'. Most preferably, each CG dinucleotide sequence present in the palindromic region of the preferred expression vectors is flanked by at least two purine

nucleotides (e.g., GA or AA) and at least two pyrimidine nucleotides (e.g., TC or TT). Examples of specific expression vector constructs useful in immunizing a host are set forth in co-pending, commonly assigned Application Serial No. 08/593,554.

Such expression vector constructs possess the advantage of stimulating cytotoxic T lymphocyte (CTL) activity to a greater degree than occurs on introduction into a host of control vectors which lack the palindromic sequences described above. In addition, those expression vectors which incorporate the flanking purine and pyrimidine nucleotides as described above also enhance stimulation of B lymphocyte activity in response to expressed initiating antigen. Thus, the expression vectors described above are preferred for their activity as immunostimulatory adjuvants to boost the host immune response to the initiating antigen which is expressed to immunize the host according to the method of the invention.

Other particularly useful recombinant expression vectors for use in the invention are those which incorporate a promoter that can be switched "on" or "off" after the vector has been administered to the patient. Use of such expression vectors in the invention aids in minimizing, if not avoiding, extracellular stimulation of IgE antibody formation against expressed asthma-initiating antigen.

Particularly efficacious examples of such promoters are the ligand inducible nuclear receptor promoters. Nuclear receptors represent a family of transcriptional enhancer factors that act by binding to specific DNA sequences found in target promoters known as response elements. Specific members of the nuclear receptor family include the primary intracellular targets for small lipid-soluble ligands, such as vitamin D₃ and retinoids, as well as steroid and thyroid hormones ("activating ligands").

Nuclear receptors activated by specific activating ligands are well suited for use as promoters in eukaryotic expression vectors since expression of genes can be regulated simply by controlling the concentration of ligand available to the receptor. For example,

glucocorticoid-inducible promoters such as that of the long terminal repeat of the mouse mammary tumor virus (MMTV) have been widely used in this regard because the glucocorticoid response elements are expressed in a wide variety of cell types. One expression system which exploits glucocorticoid response elements responsive to a wide variety of steroid hormones (e.g., dexamethasone and progesterone) is a pGREtk plasmid (containing one or more rat tyrosine amino transferase glucocorticoid response elements upstream of the herpes simplex virus thymidine kinase (tk) promoter in pBLCAT8+), transfected in HeLa cells (see, Mader and White, *Proc.Natl.Acad.Sci USA*, 90:5603-5607, 1993 [pGRE2tk]; and, Klein-Hitpass, *et al.*, *Cell*, 46:1053-1061, 1986 [pBLCAT8+]; the disclosures of which are incorporated herein by this reference to illustrate knowledge in the art concerning construction of suitable promoters derived from nuclear receptor response elements ["NRRE promoters"]). The pGREtk promoter (see, map at FIGURE 11) is particularly effective in stimulating controlled overexpression of cloned genes in eukaryotic cells (Mader and White, *supra* at 5607).

Another particularly suitable NRRE promoter for use in the invention is one which is inducible by the vitamin D₃ compound 1,25-dihydroxyvitamin D₃ and non-hypercalcemic analogs thereof (collectively, "vitamin D₃ activating ligands"). NRRE promoters inducible by vitamin D₃ activating ligands contain the vitamin D₃ receptor (VDR) response elements PurG(G/T)TCA which recognizes direct repeats separated by 3 base pairs. Vitamin D₃ response elements are found upstream of human osteocalcin and mouse osteopontin genes; transcription of these genes is activated on binding of the VDR (see, e.g., Morrison and Eisman, *J.Bone Miner.Res.*, 6:893-899, 1991; and, Ferrara, *et al.*, *J.Biol.Chem.*, 269:2971-2981, 1994, the disclosures of which are incorporated herein by this reference to illustrate knowledge in the art of vitamin D₃ responsive inducible promoters). Recent experimental results from testing of a recombinant expression vector containing the mouse osteopontin VDR upstream of a truncated herpes simplex virus thymidine kinase (tk) promoter suggested that 9-cis-retinoic acid can augment the response of VDR to 1,25-hydroxyvitamin D₃ (see, Carlberg, *et al.*, *Nature*, 361:657-660, 1993).

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Ferrara, *et al.* also described vitamin D₃ inducible promoters in recombinant expression vectors constructed using multiple copies of a strong VDR; in particular, the mouse osteopontin VDR (composed of a direct repeat of PurGTTCA motifs separated by 3 base pairs). This VDR conforms to the PurGG/TTCA consensus motifs which have previously been shown to be responsive not only to vitamin D₃, but also to thyroid hormone and/or retinoic acid. As many as three copies of the mouse VDR was inserted into pBLCAT8+; immediately upstream of the herpes simplex virus tk promoter (*see*, e.g., FIGURE 12 [map of pVDREtk]). Transfection of the resulting VDREtk vector into COS cells (producing a "VDR expression system") proved to be particularly useful in that COS cells contain the nuclear retinoid X receptor (RXR) that has been shown to act as an auxiliary factor for binding of VDR to its response element.

The VDR expression system (and functionally equivalent expression systems under the control of, for example, human osteocalcin gene promoter) is uniquely suited for use in the invention. Specifically, expression of initiating antigen administered to a mammal according to the invention by epidermal or dermal routes (particularly the former) in a vitamin D₃ responsive expression system can be switched on by topical administration of a 1,25-dihydroxyvitamin D₃ preparation at the point of entry (and off by withdrawing the vitamin D₃ preparation and/or modulated by applying or withdrawing a source of retinoic acid to or from the point of entry). Conveniently, 1,25-dihydroxyvitamin D₃ and nonhypercalcemic analogs thereof have been approved for use in topical preparations by the United States Food and Drug Administration for the treatment of psoriasis and are commercially available.

In vivo tests of the NRRE promoters in human skin indicate that they are inducible on systemic exposure to their corresponding response elements (*see*, Tsou, *et al.*, *Exp. Cell Res.*, 214:27-34, 1994 [retinoic acid activation of retinoic acid response element coupled to a Lac-Z reporter molecule in epidermis of transgenic mice]). Given the expected retention of polynucleotides administered dermally or epidermally at the point of entry (thus making them available for exposure to topically absorbed response elements; *see*,

e.g., discussion at pages 15-16 and data in Example IV), it can be reasonably predicted that use of NRRE promoters for expression of such polynucleotides will also permit their *in vivo* control through topical administration of appropriate NRRE promoter activating ligands (e.g., 1,25-dihydroxyvitamin D₃ transcriptional activators with a VDR expression vector for expression of the polynucleotide of interest).

Thus, use of an NRRE promoter recombinant expression vector for administration and expression of initiating antigens according to the invention permits control of expression to, for example, switch on expression when dosing is needed or switch off expression in the event of an adverse reaction to the expressed protein or peptide.

10 2. Pharmaceutically effective polynucleotide compositions for use in the method of the invention

Compositions of polynucleotide(s) prepared as described above may be placed into a pharmaceutically acceptable carrier for introduction into a host. The carrier chosen should not impair antigen recognition of the polynucleotide composition; for this reason, liposomal and colloidal particle-based delivery vehicles are not desirable for use in the invention. Thus, polynucleotide compositions suitable for use in the invention will most preferably consist of "naked" polynucleotides in a pharmaceutically safe carrier.

Pharmaceutically acceptable carriers may include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example,

antimicrobials, antioxidants, chelating agents, and inert gases and the like. Further, a may be lyophilized using means well known in the art, for subsequent reconstitution and use according to the invention.

Absorption promoters, detergents, chemical irritants or mechanical irritation means can enhance transmission of the polynucleotide composition through the point of entry. For reference concerning general principles regarding promoters and detergents which have been used with success in mucosal delivery of organic and peptide-based drugs, see Chien, *Novel Drug Delivery Systems*, Ch. 4 (Marcel Dekker, 1992). Specific information concerning known means and principles of nasal drug delivery are discussed in Chien, supra at Ch 5. Examples of suitable nasal absorption promoters are set forth at Ch. 5, Tables 2 and 3; milder agents are preferred. Further, known means and principles of transdermal drug delivery are also discussed in Chien, supra, at Ch. 7. Suitable agents for use in the method of this invention for mucosal/nasal delivery are also described in Chang, *et al.*, *Nasal Drug Delivery*, "Treatise on Controlled Drug Delivery", Ch. 9 and Table 3-4B thereof, (Marcel Dekker, 1992). Suitable agents which are known to enhance absorption of drugs through skin are described in Sloan, Use of Solubility Parameters from Regular Solution Theory to Describe Partitioning-Driven Processes, Ch. 5, "Prodrugs: Topical and Ocular Drug Delivery" (Marcel Dekker, 1992), and at places elsewhere in the text.

It is expected that these techniques (and others which are conventionally used to facilitate drug delivery) may be adapted to preparation of polynucleotide compositions for use in the methods of the invention by those of ordinary skill in the art without undue experimentation. In particular, although the approaches discussed in the preceding paragraphs have not, to the inventors' knowledge, been previously used for polynucleotide delivery, it is believed that they are suitable for use to that end. For that reason, the references identified above, while not essential to the inventive methods, are incorporated herein by this reference.

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C. Means For, And Routes Of, Administration of Polynucleotide Compositions.

Although it is not intended that the invention will be entirely limited by a particular theory as to the mechanism of expression involved, it is believed that a biological response in these tissues following administration of the polynucleotide compositions of the invention into skin or mucosa is achieved because the polynucleotide is expressed intracellularly in the cytoplasm of mononuclear cells, most likely the host's antigen presenting cells.

More specifically, polynucleotide compositions do not appear to be taken up directly by fibroblasts or other tissue cells in significant quantities (see, histological study in Example IV and FIGURE 4). This conclusion is borne out by studies showing that (1) intradermal administration of even minute amounts of polynucleotide compositions into mice induced a prominent Th1 response (indicative of antigen presentation by macrophages and dendritic cells; see, Examples V and VII); (2) intradermal administration of polynucleotide composition to mice induced the formation of cytotoxic T cells without stimulating production of detectable levels of antibody (see, Example VIII); and, (3) induction of prolonged immunological memory with respect to the polynucleotide expression product as an antigen (Example X). It therefore appears that the immunogenicity of polynucleotide compositions depends not on the amount of protein expressed thereby, but instead in part on the type of cell transfected (e.g., antigen presenting cells versus tissue cells).

Therefore, the ideal target tissue will be one in which approximately 1% to 2% of the cell population is comprised of antigen presenting cells; e.g., mucosa or skin. The mucosa of the respiratory tract is the primary target tissue for immunization against asthma-initiating antigens to treat allergic asthma; however, the skin may also be a target tissue for immunization against contact allergens as well as for administration of, for example, pre-immunization and booster doses of antigen as well as other therapeutically significant peptides. In addition, because IgE molecules are predominately present in

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mucosa and skin, use of these routes as points of entry according to the invention can be expected to be particularly effective in moderating allergic responses to antigen.

For use in primary immunizations against asthma-initiating antigens, intranasal administration means are most preferred. These means include inhalation of aerosol
5 suspensions or insufflation of the polynucleotide compositions of the invention. Nebulizer devices suitable for delivery of polynucleotide compositions to the nasal mucosa are well-known in the art and will therefore not be described in detail here.

To enhance absorption of the polynucleotide compositions of the invention, the compositions may include absorption promoters and/or detergents described in Section
10 II-B, *supra*. To increase the population of APC's at the site of entry, a chemical irritant may also be employed.

In particular, the polynucleotides may include a chemical which irritates the outermost epithelial cells of the mucosa, thus provoking a sufficient immune response to attract
15 additional APC's to the area. An example is a keratinolytic agent, such as the salicylic acid used in the commercially available topical depilatory creme sold by Noxema Corporation under the trademark NAIR.

Dermal routes of administration, as well as subcutaneous injections, are useful in co-administration of other therapeutically significant peptides, as well as in immunizations
20 and antigen boosters. The means of introduction for dermal routes of administration which are most preferred are those which are least invasive. Preferred among these means are transdermal transmission and epidermal administration.

For transdermal transmission, iontophoresis is a suitable method. Iontophoretic transmission may be accomplished using commercially available "patches" which deliver
25 their product continuously through unbroken skin for periods of several days or more. Use of this method allows for controlled transmission of pharmaceutical compositions

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in relatively great concentrations, permits infusion of combination drugs and allows for contemporaneous use of an absorption promoter.

An exemplary patch product for use in this method is the LECTRO PATCH trademarked product of General Medical Company of Los Angeles, CA. This product electronically maintains reservoir electrodes at neutral pH and can be adapted to provide dosages of differing concentrations, to dose continuously and/or to dose periodically. Preparation and use of the patch should be performed according to the manufacturer's printed instructions which accompany the LECTRO PATCH product; those instructions are incorporated herein by this reference.

10 Epidermal administration essentially involves mechanically or chemically irritating the outermost layer of the epidermis sufficiently to provoke an immune response to the irritant. Specifically, the irritation should be sufficient to attract APC's to the site of irritation. As discussed previously, it is believed that the APC's then take up and express the administered polynucleotide composition.

15 Alternatively, additional APCs can be attracted to the point of entry with mechanical irritant means. An exemplary mechanical irritant means employs a multiplicity of very narrow diameter, short tynes which can be used to irritate the skin and attract APC's to the site of irritation, to take up polynucleotide compositions transferred from the end of the tynes. For example, the MONO-VACC old tuberculin test manufactured by Pasteur Merieux of Lyon, France contains a device suitable for introduction of polynucleotide compositions.

The device (which is distributed in the U.S. by Connaught Laboratories, Inc. of Swiftwater, PA) consists of a plastic container having a syringe plunger at one end and a tyne disk at the other. The tyne disk supports a multiplicity of narrow diameter tynes of a length which will just scratch the outermost layer of epidermal cells. Each of the tynes in the MONO-VACC kit is coated with old tuberculin; in the present invention,

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each needle is coated with a pharmaceutically effective polynucleotide composition or a mixture thereof. Use of the device is according to the manufacturer's written instructions included with the device product; these instructions regarding use and administration are incorporated herein by this reference to illustrate conventional use of the device. Similar devices which may also be used in this embodiment are those which are currently used to perform allergy tests.

D. Dosing Parameters for the Polynucleotide Compositions of the Invention

As noted above, it is probable that introduction of relatively low doses of asthma-initiating antigen-encoding polynucleotide to APC's using the method of the invention assists the asthma-initiating antigen in being expressed and retained intracellularly, thus limiting the extracellular availability of the asthma-initiating antigen for stimulation of IgE antibody production and formation of asthma-initiating antigen/IgE antibody complexes. Conversely, it appears that introduction of relatively "high" doses of asthma-initiating antigen-encoding polynucleotides (e.g., substantially greater than about 50µg in mice) can stimulate production of IgE antibody at levels that are more comparable to those produced in mice injected subcutaneously with an asthma-initiating antigen, possibly due to extracellular release of antigen.

Thus, the preferred embodiment of the method for treatment of allergies of the invention will be one in which the asthma-initiating antigen-encoding polynucleotide is administered in "low" doses (e.g., preferably less than about 50µg of polynucleotide in mice). Those of ordinary skill in the art will readily be able to determine an equivalent dosage level for use in humans. Those of ordinary skill in the art will be familiar with the course of dosing employed in asthma-initiating antigen immunotherapy (i.e., priming, booster and maintenance dosing), which course will be suitable for use in the method of the invention. Generally, it can be expected that murine equivalent doses of less than about 50µg, and even less than about 10µg, will be suitable for priming, booster and maintenance doses in humans.

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Alternatively, the priming dose of asthma-initiating antigen-encoding polynucleotide may be followed by booster and/or maintenance doses of asthma-initiating antigen. Once an immunologic memory regarding the asthma-initiating antigen has been induced through introduction of an asthma-initiating antigen-encoding polynucleotide, that
5 memory is maintained despite subsequent asthma-initiating antigen challenge.

Advantageously, because a polynucleotide that will operatively encode for an antigen is administered in lieu of the antigen itself, the quantity of foreign material being introduced to the host is relatively minimal. Moreover, routes of administration of polynucleotide compositions through skin or mucosa require a lower concentration of
10 DNA to produce the same magnitude of immune response than does, for example, the intramuscular route of administration for the same compositions (e.g., about 10-50 fold lower; see, e.g., Example X). As a result, the invention lends itself well to the administration of polynucleotide compositions which code for up to several hundred different antigens for use in immunizing a host against more than one asthma-initiating
15 antigen at a time. Thus, the invention also encompasses the administration of a peptide cocktail (i.e., mixture of polynucleotides) via expression of gene constructs containing, for example, up to 200 asthma-initiating antigen encoding polynucleotide sequences under the control of a single promoter.

Means to confirm the presence and quantity of expressed peptides are well-known to
20 those skilled in the art and will not, therefore, be described in detail. Certain such means are illustrated in the Examples provided below; generally, they include immunoassays (such as enzyme-linked immunoabsorbent assays), PCR techniques, and immunohisto-logical analyses performed according to techniques which are well known in the art. Dosages of the administered polynucleotides can be adjusted to achieve the desired level
25 of expression based on information provided by these detection and quantification means as well as *in vivo* clinical signs known to practitioners skilled in the clinical arts.

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Examples illustrating aspects of each embodiment of the invention are provided below. They should be regarded as illustrating rather than limiting the invention.

EXAMPLE I

MURINE MODEL FOR THE AIRWAY HYPERREACTIVITY OF ALLERGIC ASTHMA

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Aeroasthma-initiating antigen challenged mice of different strains model the airway hyperreactivity seen in allergic asthma. Suitable murine strains for use in modeling the disease include Balb/c mice (which are IL-5+ and produce enhanced concentrations of IL-4 in response to CD4+ lymphocyte priming), C57BL/6 mice (which are IL-5
10 deficient, for detailed study of IL-5 induced tissue damage in asthma) and *W/W^v* mice (which are mast cell deficient, for detailed study of mast cell activation in asthma).

Disease modeling mice are conveniently prepared by intraperitoneal or subcutaneous injection of ovalbumin ("OVA") in carrier (e.g., sterile saline), followed by antigen challenge with aerosolized antigen. For example, mice may be immunized with 25µg
15 OVA by subcutaneous injection (with or without adjuvant) weekly for 4-6 weeks, then challenged with 2 or 3 weekly aerosolizations of OVA at a concentration of 50 mg/ml in phosphate buffered saline (PBS) delivered in 20 minute intervals or at a concentration of 10 mg/ml 0.9% saline daily for about a week (in three 30 minute intervals daily). Nebulizer devices for use in the aerosolization are available from Aerotech II, CIS-US,
20 Bedford, MA), with a nasal chamber adapted for murine nasal passages (e.g., a nose-only chamber from Intox Products, Albuquerque, NM). When driven by compressed air at a rate of 10 liters/min., the devices described produce aerosol particles having a median aerodynamic diameter of 1.4µm.

Control mice are preferably littermates which are protein-antigen challenged without
25 prior immunization. For further details concerning this animal model, those of skill in

the art may wish to refer to Foster, *et al.*, *J.Exp.Med.*, 195-201, 1995; and, Corry, *et al.*, *J.Exp.Med.*, 109-117, 1996.

EXAMPLE II

REDUCTION OF EOSINOPHIL ACCUMULATION IN LUNG TISSUE IN A MURINE ASTHMA MODEL

5

Three sets of 3-4 C57BL/6 mice, 6-10 weeks of age, were prepared as models of allergic asthma as described in Example I (subcutaneous injection of OVA followed by antigen challenge at a concentration of 50mg OVA/ml PBS). Prior to immunization according to this scheme, two sets of the mice were pre-immunized with plasmid expression vectors which included genes for soluble OVA and ampicillin resistance (AmpR). One of the sets of pre-immunized mice received plasmids which encoded a chimeric antigen expressed as a product of fusion between the gene encoding OVA and the gene for the transferrin receptor. This "fusion" plasmid directs the production of a transmembrane, non-secreted protein at the site of immunization. Pre-immunization was performed by subcutaneous injection as described in Example VII.

15

On days 0, 7, 14 and 21, the two sets of pre-immunized mice and set of control mice were injected subcutaneously with 25µg of OVA in 0.2ml PBS. On days 26 and 31, each mouse was nebulized with 10ml of 50mg OVA/ml PBS using the nebulizer device described in Example I.

On day 32, each mouse was bled by tail snip (approximately 50µl volume) into a 0.1mM solution of PBS and EDTA. Red blood cells in solution were lysed with 150mM NH₄Cl and 10mM KHCO₃ in dH₂O then stained (Wright-Giesma stain). Lung lavage from each mouse was obtained after sacrifice by canalization of the trachea and lavage with 800 microliters PBS, then the lavage product was stained. Bone marrow samples from each mouse were obtained by flushing of extracted femur marrow with PBS. Histological specimens of lung and trachea tissue were obtained from the right lower lobe of the lung

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and trachea. Specimens were frozen, sectioned to a 5 micron width and stained with DAB peroxidase.

Eosinophil counts (with a single count=at least 300 cells) were obtained in each sample from each mouse. Results are expressed in the Table below as percent eosinophils compared to total leukocytes in each sample. In summary, the control mice (nos. 1-4) had an average of 41.3% eosinophils in the lung/trachea tissue samples. In contrast, the mice pre-immunized with the soluble OVA encoding plasmid (nos. 53-56 and 57-60) had 50% less eosinophil accumulation in these tissues compared to the control mice. Interestingly, the mice pre-immunized with the chimeric antigen encoding plasmid (nos. 65-68) had at least 90% reduction in eosinophil accumulation in these tissues as compared to the control mice. These data indicate that the IL-4 and IL-5 stimulated eosinophil accumulation in lung tissue which characterizes the late phase of allergic asthma is inhibited by polynucleotide immunization according to the immunization scheme of the invention.

TABLE 1

| Mouse # | Bone Marrow | Peripheral blood | Bronchoalveolar Lavage |
|---------|---------------|------------------|------------------------|
| 1 | 9.3 | 2.0 | 47.4 |
| 2 | 6.0 >avg 10.3 | 4.4 >avg 5.0 | 65.0 >avg 43.1 |
| 3 | 14.3 | 12.1 | 24.2 |
| 4 | 11.4 | 1.4 | 35.7 |
| 53 | 0.3 | 3.6 | 45.0 |
| 54 | 4.2 >avg 3.9 | 5.8 >avg 4.2 | 20.4 >avg 25.4 |
| 55 | 0.8 | 2.5 | 10.9 |
| 56 | 10.2 | 4.8 | (trachea damaged) |
| 57 | 1.5 | 1.3 | 2.7 |
| 58 | 1.5 >avg 1.4 | 5.2 >avg 2.3 | 2.2 >avg 3.5 |
| 59 | 1.9 | 1.2 | 1.3 |
| 60 | 0.6 | 1.5 | 7.8 |
| 65 | 3.2 | 1.2 | 14.2 |
| 66 | 4.4 >avg 3.8 | 3.5 >avg 1.9 | 31.0 >avg 21.3 |
| 67 | 4.9 | 1.7 | 34.1 |
| 68 | 2.7 | 1.2 | 5.8 |

EXAMPLE III

**GENE EXPRESSION FOLLOWING
INTRANASAL INTRODUCTION OF A POLYNUCLEOTIDE
COMPOSITION**

To test the level of protein expression following intranasal introduction of a polynucleotide composition (here, one to express influenza ribonucleoprotein from a plasmid under the control of a CMV promoter) was introduced to Balb/c mice in 3

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groups of 6 intranasally. Levels of anti-NP IgG in peripheral blood before and after introduction of the plasmid at various serum dilutions were measured by ELISA as described in Example II. Blood was drawn from each mouse after intranasal introduction after 6 weeks.

5 FIGURES 1-3 graphically depict the results of the ELISA assays before and after intranasal introduction of the plasmid. The graphs plot ELISA titer against serum dilution. In FIGURE 1, values are shown for individual mice from each group (#1-3) and an average value from all mice in each group (#G1-G3).

Without anesthesia, mice in a second group which received $3 \times 7.5 \mu\text{g}$ of plasmid showed
10 enhanced titers of antibody as compared to background (FIGURE 1). These data are shown in FIGURE 2.

A third group of mice received the same gravity of plasmid under anesthesia. Expression of RNP as indicated by titers of anti-NP IgG in these mice was substantially similar to the expression achieved in the unanesthetized mice. The data for the anesthetized mice
15 are shown in FIGURE 3.

Expression can be enhanced by additional use of absorption promoters, and prolonged by time-released promoters whose identity and use are known in the art such as those suggested in Chien, *supra*, at Ch. 5.

EXAMPLE IV

20 **HISTOLOGICAL STUDIES SHOWING CELL UPTAKE
OF POLYNUCLEOTIDE COMPOSITIONS BY MONONUCLEAR CELLS
AT THE POINT OF ENTRY IN SKIN**

Three days after intradermal injection of the tails of naked pCMV-*lacZ* (encoding β -galactosidase) into Balb/c mice, the mice were sacrificed. Tissue cultures were obtained

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at the point of entry for the plasmid and stained for *E. coli* β -galactosidase activity. A photograph (40x magnification) of a slide from the histological examination of these cultures is contained in FIGURE 4.

As shown in FIGURE 4, uptake of the plasmid is shown (in blue) to be by mononuclear cells. The fibroblasts in the tissue samples are not stained, thus indicating that the plasmid was not taken up by these cells. The rounded, mononuclear cells which did take up the plasmid appear to be macrophages and/or other antigen presenting cells, which would indicate that uptake of the plasmid is by phagocytosis.

EXAMPLE V

10 **SELECTIVE INDUCTION OF A Th1 RESPONSE AFTER ADMINISTRATION OF POLYNUCLEOTIDE COMPOSITIONS ACCORDING TO THE INVENTION**

In mice, IgG 2A antibodies are serological markers for a Th1 type immune response, whereas IgG 1 antibodies are indicative of a Th2 type immune response. Th2 responses include the allergy-associated IgE antibody class; soluble protein antigens tend to stimulate relatively strong Th2 responses. In contrast, Th1 responses are induced by antigen binding to macrophages and dendritic cells. Th1 responses are to be of particular importance in the treatment of allergies and AIDS.

To determine which response, if any, would be produced by mice who received polynucleotide compositions according to the invention, mice were vaccinated with pCMV Lac-Z or protein as described in the preceding example. At 2 week intervals, any IgG 2a and IgG 1 to β -galactosidase were measured by enzyme-linked immunoabsorbent assay (using antibodies specific for the IgG 1 and IgG 2A subclasses) on microtiter plates coated with the enzyme.

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As shown in FIGURE 5, only the mice who received the plasmid by ID injection produced high titers of IgG 2A antibodies. As shown in FIGURE 6, immunization of the mice with the enzyme itself ("PR") induced production of relatively high titers of IgG 1 antibodies. In the IM injected mice, low titers of both IgG 2A and IgG 1 antibodies were produced without apparent selectivity. The data shown in the FIGURES comprise averages of the values obtained from each group of 4 mice.

To determine the stability of the antibody response over time, the same group of animals were boosted with 0.5 μ g of enzyme injected intradermally. As shown in FIGURES 7 and 8 boosting of ID injection primed animals with the enzyme induced a nearly 10-fold rise in IgG 2A antibody responses (i.e., the antibody titer rose from 1:640 to 1:5120), but did not stimulate an IgG 1 response. These data indicate that the selective Th1 response induced by ID administration of polynucleotide compositions is maintained in the host, despite subsequent exposure to antigen.

EXAMPLE VI

Th1 RESPONSES IN MICE AFTER ADMINISTRATION OF POLYNUCLEOTIDE COMPOSITIONS WITH A MECHANICAL IRRITANT

The experiments described in Example V were repeated in separate groups of mice, except that (1) only a priming dose was tested, and (2) the pCMV Lac-Z plasmid was administered to one group of 4 mice using the MONO-VACC® tyne device described in the disclosure, while β -galactosidase protein (10 μ g) was administered to another group of 4 mice by intradermal (ID) injection.

As shown in FIGURE 9, the mice who received plasmid produced relatively low titers of IgG 1 antibody compared to the mice who received the protein. In contrast, as shown in FIGURE 10, the mice who received plasmid produced substantially higher titers of IgG 2A antibody as compared to the mice who received the protein.

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Interestingly, the mice who received the plasmid via scratching of their skin with the tyne device produced even higher titers of IgG 2A antibody than did the mice who received the same plasmid via ID injection (both of which groups produced higher titers of IgG 2A antibody than did the mice who received the plasmid via IM injection). These results indicate that scratching of skin with the tyne device attracts greater number of APC's to the "injured" point of entry for the polynucleotide compositions and are consistent with the theory that APC's are more efficient targets for gene administration and expression than are muscle or other somatic cells.

The data shown in the FIGURES comprise averages of the values obtained from each group of 4 mice.

EXAMPLE VII

SUPPRESSION OF IgE ANTIBODY RESPONSE TO ANTIGEN BY IMMUNIZATION WITH ANTIGEN-ENCODING POLYNUCLEOTIDES

Five to eight week old Balb/c mice were immunized with one of two recombinant expression vectors: pCMV-Lac-Z or a control plasmid, pCMV-BL (which does not encode for any insert peptide). A third group of the mice received injections of antigen (β galactosidase). Plasmid DNA was purified and its endotoxin content reduced to 0.5-5ng/1mg DNA by extraction with TRITON X-114 (Sigma, St. Louis, MI). Before inoculation, pDNA was precipitated in ethanol, washed with 70% ethanol and dissolved in pyrogen free normal saline.

Immunization was by intradermal injection of plasmid DNA loaded onto separate tynes of a MONO-VACC® multiple tyne device (Connaught Lab, Inc., Swiftwater, PA). Briefly, the tyne devices were prepared after extensive washing in DDW and overnight soaking in 0.5% SDS (sulfated dodecyl saline), washed again in DDW, soaked overnight in 0.1N NaOH, washed again in DDW and dried at 37°C for 8 hours. Six μ l of plasmid

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DNA dissolved in normal saline were pipetted onto the tynes of the tyne device just prior to each inoculation described below. The total amount of pDNA loaded on the device per inoculation was 25 µg each of pCMV-Lac-Z and pCMV-BL. For purposes of estimating actual doses, it was assumed that less than 10% of the pDNA solution loaded
5 onto the tyne device was actually introduced on injection of the tynes into intradermal tissue.

Each mouse was treated 3 times with 2 inoculations of each plasmid in a one week interval injected intradermally at the base of the tail. Another group of mice received a single intradermal injection in the base of the tail of 10µg of β galactosidase protein
10 (dissolved in 50µl of normal saline) in lieu of pDNA.

Toward inducing an IgE antibody response to subsequent asthma-initiating antigen challenge, each group of mice was injected once intraperitoneally with 0.1 ml of phosphate buffered saline (PBS) solution containing 1µg of antigen (β galactosidase; Calbiochem, San Diego, CA) and 3mg of ALUM aluminum hydroxide as adjuvant
15 (Pierce Chemical, Rockford, IL) 14 weeks after the initial immunization. Total IgE was assayed in sera from the mice 4 times over the subsequent 4 consecutive weeks.

IgE was detected using a solid phase radioimmunoassay (RAST) in a 96 well polyvinyl plate (a radioisotopic modification of the ELISA procedure described in Coligan, "Current Protocols In Immunology", Unit 7.12.4, Vol. 1, Wiley & Sons, 1994), except
20 that purified polyclonal goat antibodies specific for mouse ε chains were used in lieu of antibodies specific for human Fab. To detect anti-Lac-Z IgE, the plates were coated with β galactosidase (10µg/ml). The lowest IgE concentration measurable by the assay employed was 0.4ng of IgE/ml.

As shown in FIGURE 13, mice injected with pCMV-Lac-Z produced only low levels of
25 total IgE antibody (averaging about 250 CPM in RAST) as compared to mice injected with β galactosidase (averaging about 1000 CPM in RAST). Moreover, IgE levels in the

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plasmid injected mice remained consistently low (averaging about 250-450 CPM) despite boosting with protein (indicating that tolerance was acquired in these mice on initial immunization), while IgE levels in the protein injected mice rose substantially (averaging about 1500 to 2000 CPM) after boosting, then eventually tapered off to control levels at week 4 as tolerance was acquired by the protein injected mice through repeated exposure to the protein antigen.

Measuring specifically the anti-antigen response by each group of mice, as shown in FIGURE 14, anti-Lac-Z IgE levels in the plasmid injected mice again were consistently low both before and after boosting (averaging about 250 CPM in RAST), while the protein injected mice developed high levels of anti-Lac-Z, particularly after the first antigen booster injection, when anti-Lac-Z levels in the mice rose to an average of about 3000 CPM. Consistent with acquisition of tolerance, anti-Lac-Z IgE levels in the protein injected mice declined over time, but continued to rise in the control mice who had not received any immunization to β galactosidase.

These data show that the plasmid injected mice developed an antigen specific Th1 response to the plasmid expression product with concomitant suppression of IgE production, while tolerance was acquired in the protein injected mice only after development of substantially higher levels of total and antigen specific IgE antibodies.

EXAMPLE VIII

IL-4 AND $\text{INF}\gamma$ LEVELS IN MICE AFTER IMMUNIZATION WITH ANTIGEN OR ANTIGEN-ENCODING POLYNUCLEOTIDES

To confirm that the results shown by the data presented in Examples V through VII can be attributed to the selective induction of Th1 responses (e.g., $\text{INF}\gamma$ secretion) in plasmid injected mice (which responses are believed to exert a negative effect on IgE stimulatory Th2 responses; e.g., secretion of IL-2), levels of IL-2 and $\text{INF}\gamma$ were assayed in the sera

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of the plasmid and protein injected mice of Example VII at week one, after one booster injection of antigen. IL-2 levels were assayed using a commercial kit; INF γ levels were assayed with an anti-INF γ murine antibody assay (see, e.g., Coligan, "Current Protocols in Immunology", Unit 6.9.5., Vol. 1, Wiley & Sons, 1994).

- 5 As shown in FIGURE 15, levels of IgE stimulatory IL-4 in the protein injected mice were substantially higher than in plasmid injected mice (by about a 9:1 ratio). Conversely, levels of INF γ in the plasmid injected mice were substantially higher than in the protein injected mice (by a ratio of about 11:1).

EXAMPLE IX

10 PRODUCTION AND MAINTENANCE OF CYTOTOXIC T LYMPHOCYTES AFTER IMMUNIZATION WITH ANTIGEN OR ANTIGEN-ENCODING POLYNUCLEOTIDES

- As discussed elsewhere above, it is believed that cytotoxic T lymphocytes (CTLs) suppress Th2 cell activity, which in turn would suppress the ability of such cells to
15 stimulate the development of IgE antibodies. To confirm whether the plasmid injected mice developed CTL's and maintained the anti-antigen protection afforded thereby, CTL levels in plasmid injected and control mice were measured.

- The plasmid injected mice were immunized with a plasmid encoding influenza ribonucleoprotein. Control mice received a plasmid that did not code for an insert peptide
20 (pCMV-BL). The total amount of pDNA loaded on the tyne device per inoculation was 50 μ g of pCMV-NP and 25 μ g of pCMV-BL.

36 weeks after immunization, the mice were sacrificed and splenocytes were removed for use in standard mixed lymphocyte cultures. The cultures were grown in the presence of a known synthetic peptide representing the major H-2^d restricted CTL epitope of the

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NP protein. The cultures were assayed for anti-NP CTL activity 5-6 days later using NP peptide pulsed syngeneic P815 tumor cells (ATCC # TIB64, Rockville, MD) as targets.

As shown in FIGURE 16, mixed lymphocyte cultures prepared from the pCMV-NP injected animals displayed high levels of specific anti-NP cytolytic activity, reaching 5 10%, 30% and 80% of specific lysis at an effector to target (E/T) ratio of 5:1, 15:1 and 45:1, respectively. Control mice only displayed 1%, 1% and 9% under the same conditions. Further, in absence of exposure to the H-2^d epitope peptide, there were not significant differences in CTL activity in the pCMV-NP injected and control mice (FIGURE 17). These data indicate selective activation of Th1 cells in the pCMV-NP
10 injected mice.

EXAMPLE X

PROLONGED IMMUNOLOGIC MEMORY INDUCED BY ANTIGEN STIMULATION OF T CELLS AFTER ADMINISTRATION OF POLYNUCLEOTIDE COMPOSITIONS

15 0.1, 1, 10 and 100 µg of polynucleotide compositions in plasmid form (0.5-5 ng/1 mg DNA endotoxin content) encoding the *E.coli* enzyme β-galactosidase under the control of the CMV promoter ("pCMV Lac-Z") were administered to groups of 4 mice\dosage\route either intramuscularly ("IM") or intradermally ("ID"). For comparison, another group of 4 mice\dosage received 100 µg β-galactosidase protein
20 ("PR") intradermally. All injections were made using 50 µl normal saline as carrier. IM and ID injections were made with a 0.5 ml syringe and a 28.5 gauge needle. Antibodies were thereafter measured by enzyme-linked immunoabsorbent assay at 2 week intervals.

Briefly, total antibodies were measured using β-galactosidase (Calbiochem, CA) as the solid phase antigen. Microtiter plates (Costar, Cambridge, MA) were coated with 5 µg
25 of antigen dissolved in 90mM borate (pH 8.3) and 89mM NaCl (i.e., borate buffered

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saline; BBS) overnight at room temperature and blocked overnight with 10 mg/ml of bovine serum albumin in BBS.

Serum samples were serially diluted in BBS starting at a 1:40 dilution for the first 8 weeks, then a 1:320 dilution thereafter. These samples were added to the plates and
5 stored overnight at room temperature. Plates were washed in BBS+0.05% polysorbate 20, then reacted with a 1:2000 dilution of alkaline phosphatase labeled goat anti-mouse IgG antibody (Jackson Immunoresearch Labs., West Grove, PA) for 1 hour at room temperature, or were reacted with a 1:2000 dilution of alkaline phosphatase labeled goat anti-mouse IgG 1 antibody (Southern Biotech of AL), or were reacted with a 1:500
10 dilution of alkaline phosphatase labeled rat anti-mouse IgG 2A antibody (Pharmingen, of CA), under the same conditions. Plates were washed again, then a solution of 1 mg/ml of p-nitrophenol phosphate (Boehringer-Mannheim, Indianapolis, IN) in 0.05 M carbonate buffer (pH 9.8), containing 1mM MgCl₂ was added. Absorbance at 405 nm was read 1 hour after addition of substrate to the plates.

15 As shown in FIGURE 18, antibody responses of equivalent magnitude were induced in the animals who had received the pCMV Lac-Z plasmids by ID injection and the animals who had received the PR, while lesser antibody responses were measured in the animals who had received the pCMV Lac-Z plasmids by IM injection.

To assess for T cell memory, the animals were then boosted with 0.5 µg of PR at a
20 separate site by ID injection. If these animals had developed memory T cells to control production of antibody to β-galactosidase, they would be expected to mount a more vigorous immune response after boosting with soluble protein antigen than had been demonstrated in response to the priming dose of antigen.

As shown in FIGURE 19, it is clear that the animals which had received ID injections
25 of pCMV Lac-Z plasmid had developed substantially better immunological memory than did animals which had received either IM injections of plasmid or of PR. Further, the

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memory which was developed by the ID injected animals persisted for a minimum of about 12 weeks.

The invention claimed is:

CLAIMS

1. A method for treating both the early and late phases of allergic asthma in a host comprising:

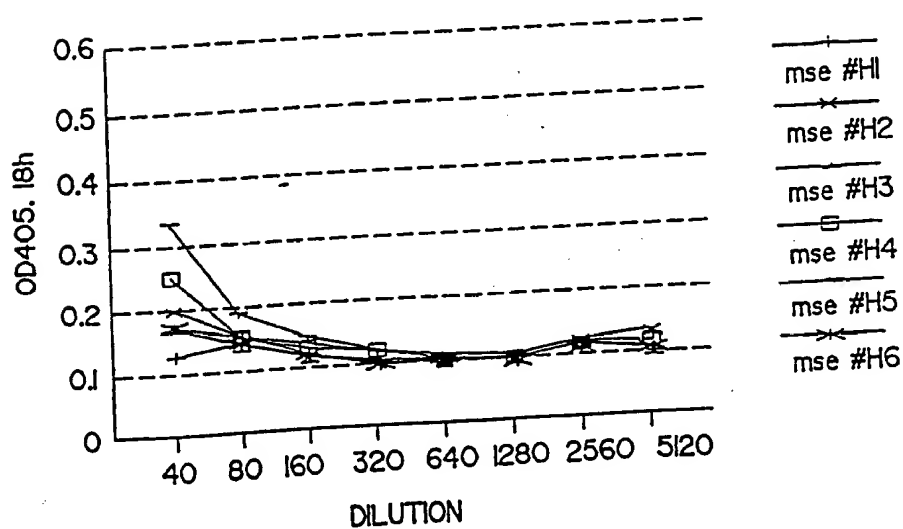
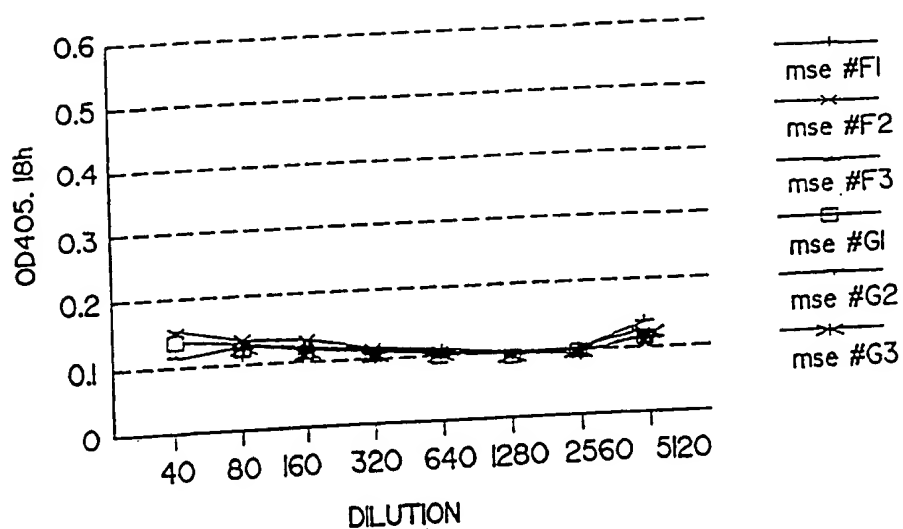
immunizing the host against an asthma-initiating antigen by introducing a
5 naked polynucleotide composition comprised of a recombinant expression vector which encodes an asthma-initiating antigen into the host's mucosa or skin, which tissues contain a relatively high concentration of antigen presenting cells as compared to other host tissues;

10 wherein the asthma-initiating antigen is expressed in the antigen presenting cells without substantial secretion therefrom to preferentially stimulate Th1 lymphocytes in the host to suppress antigen-stimulated IgE antibody production as well as IL-4 and IL-5 mediated eosinophil infiltration of lung tissue.

15
2. The method according to Claim 1 wherein the polynucleotide composition is administered in a dose equivalent to a dosage in a mouse of about 50µg polynucleotide or less.
3. The method according to Claim 1 wherein the polynucleotide composition is
20 introduced into the respiratory passages of the host.
4. The method according to Claim 3 wherein the naked polynucleotide composition is aerosolized.

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5. The method according to Claim 1 wherein the naked polynucleotide composition is coated onto the tynes of a multiple tyne device and is administered by penetrating the skin of the host with the tynes.



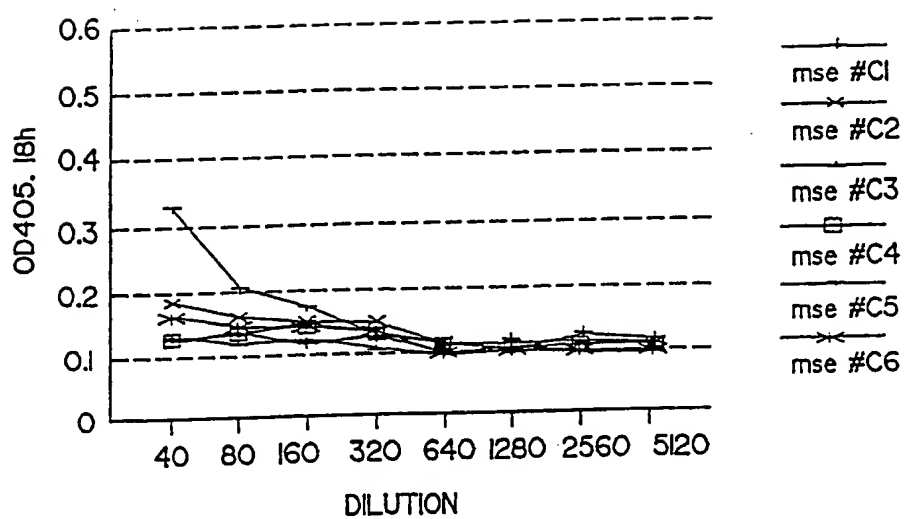


FIG. 3



FIG. 4

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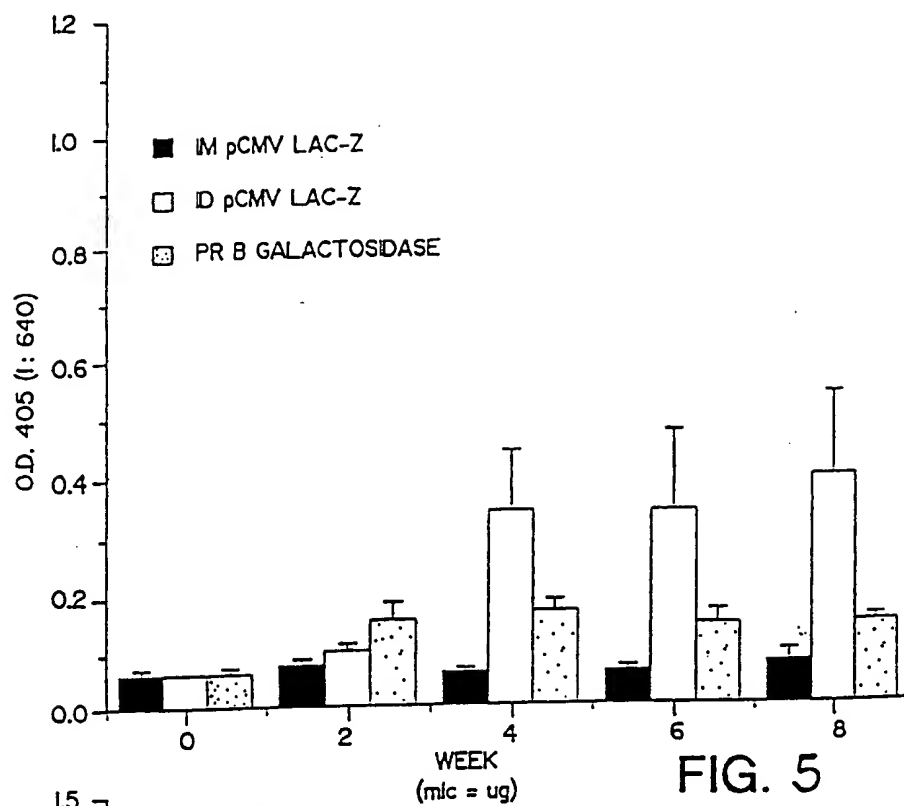


FIG. 5

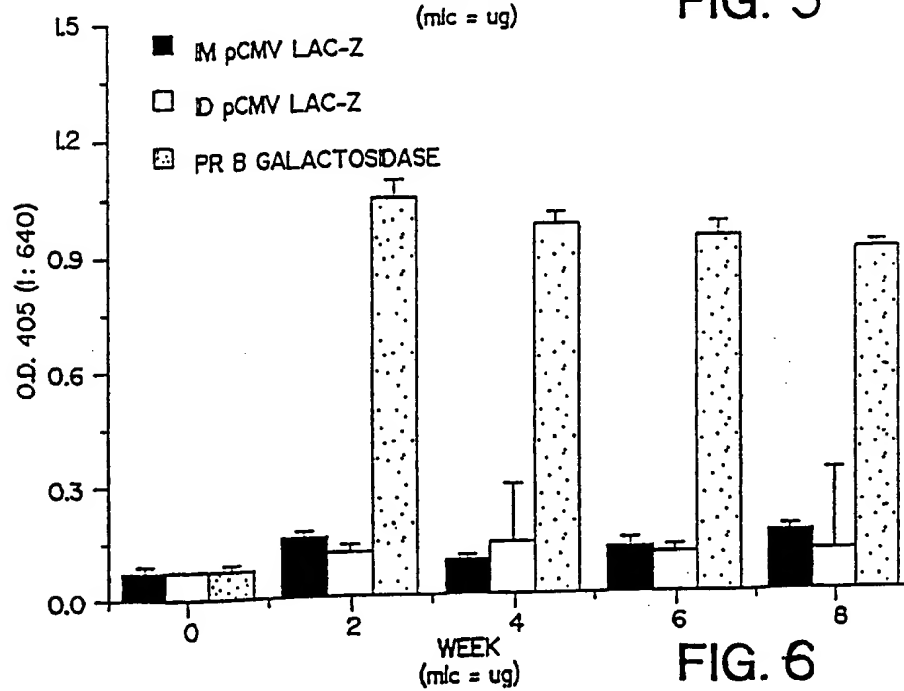


FIG. 6

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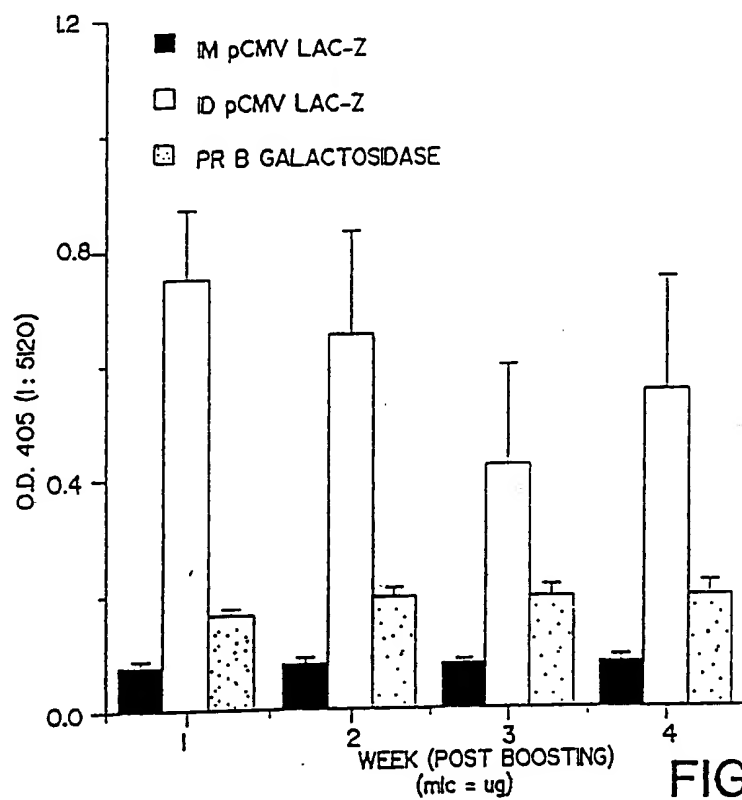


FIG. 7

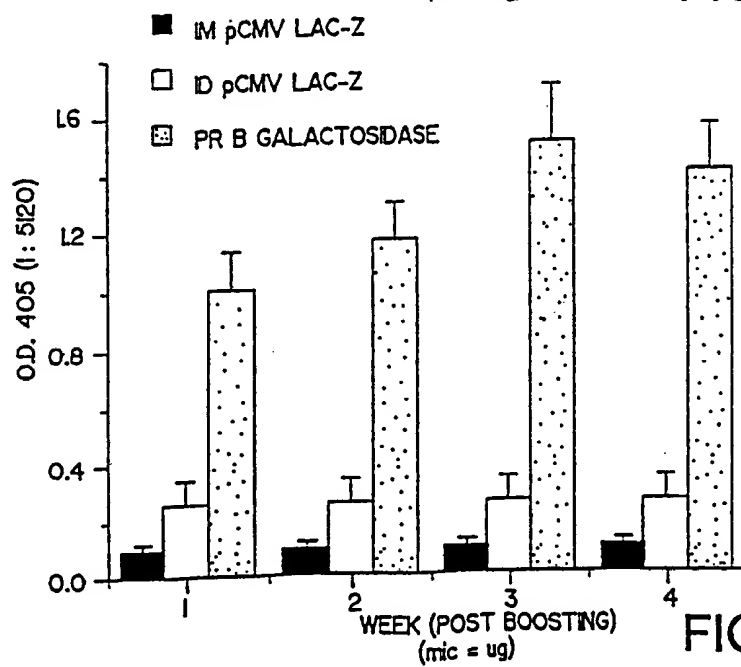


FIG. 8

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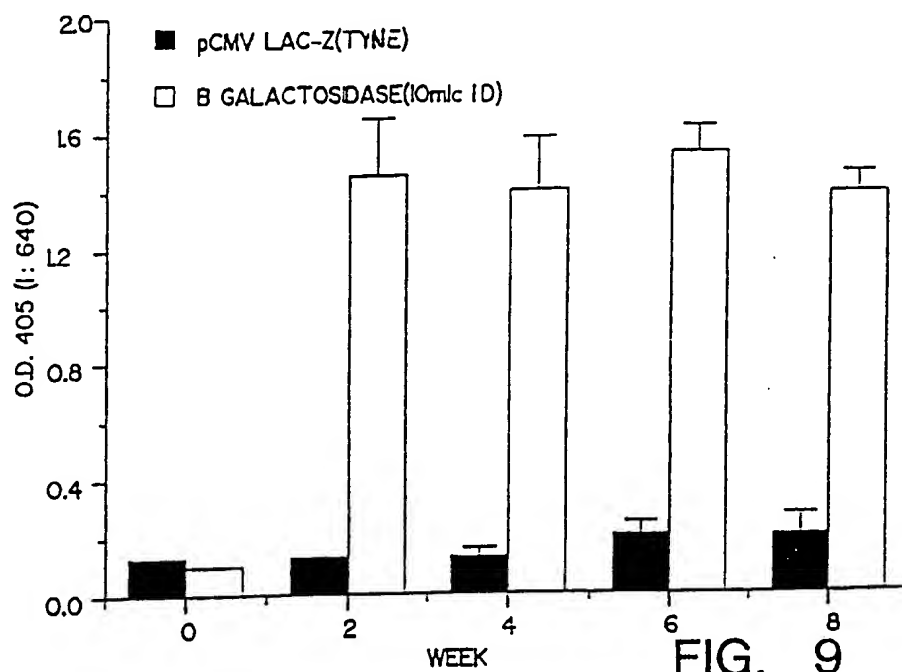


FIG. 9

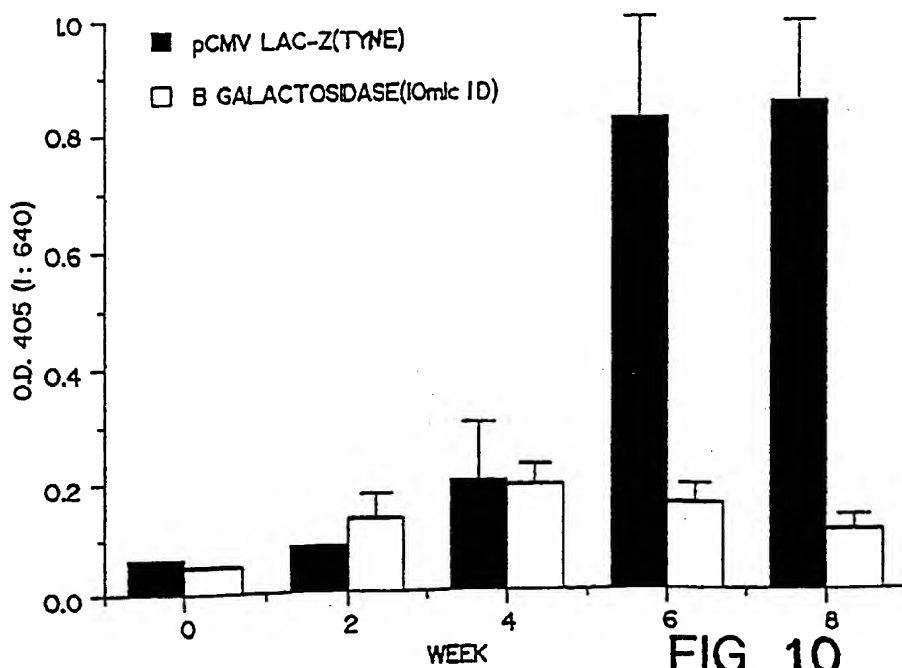


FIG. 10

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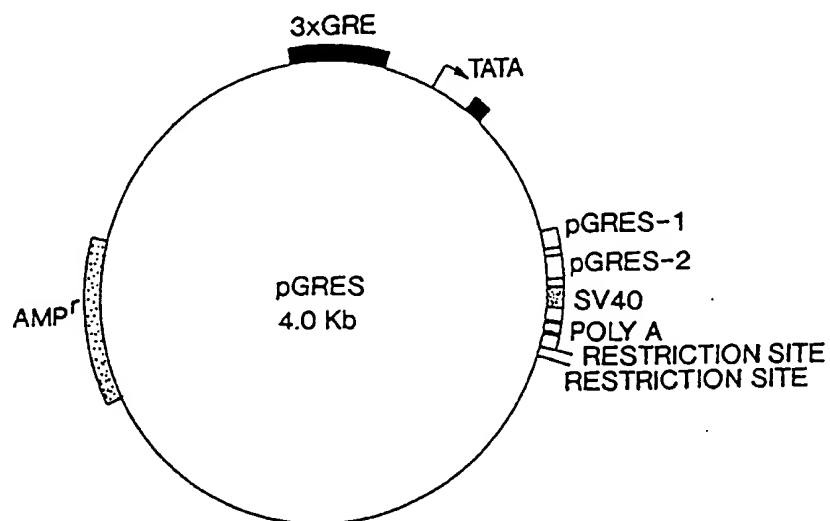


FIG. 11

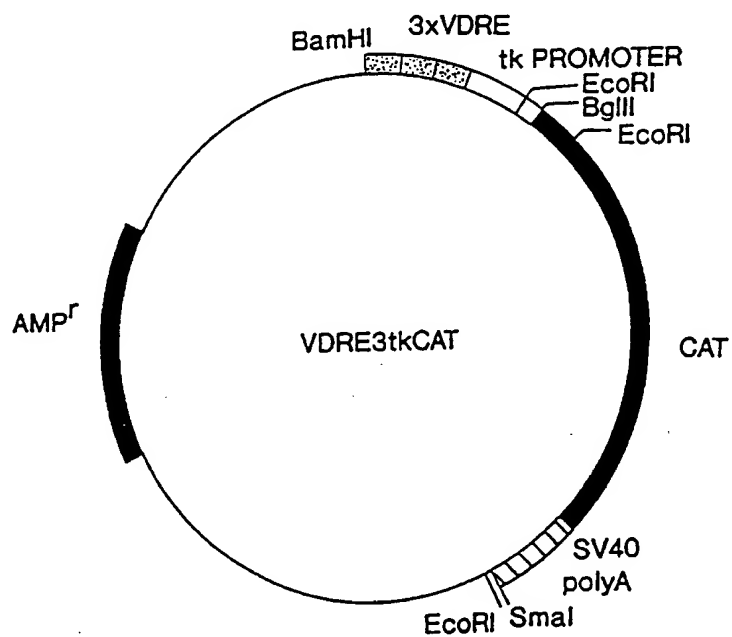


FIG. 12

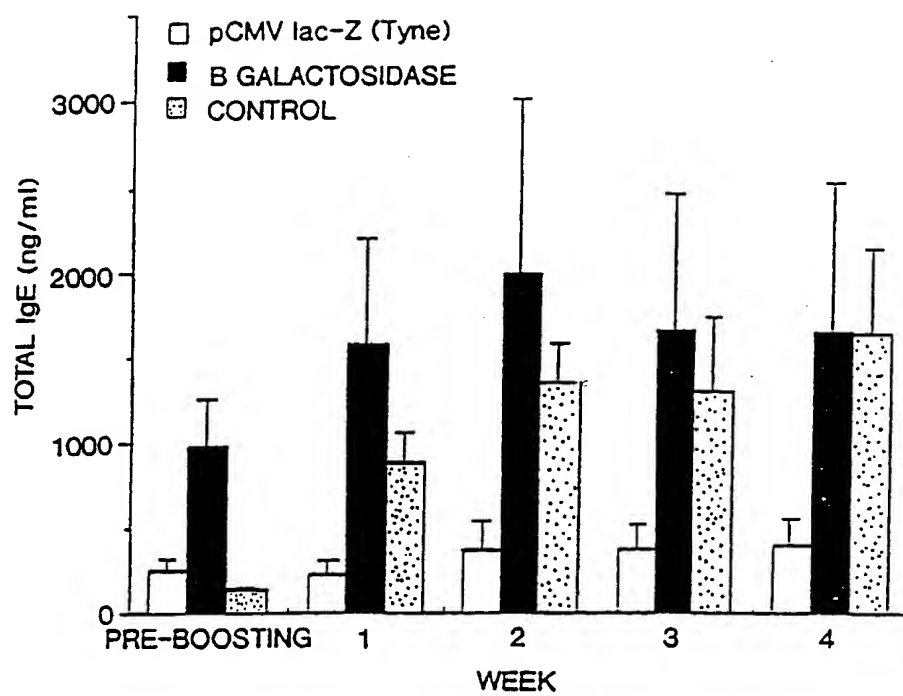


FIG. 13

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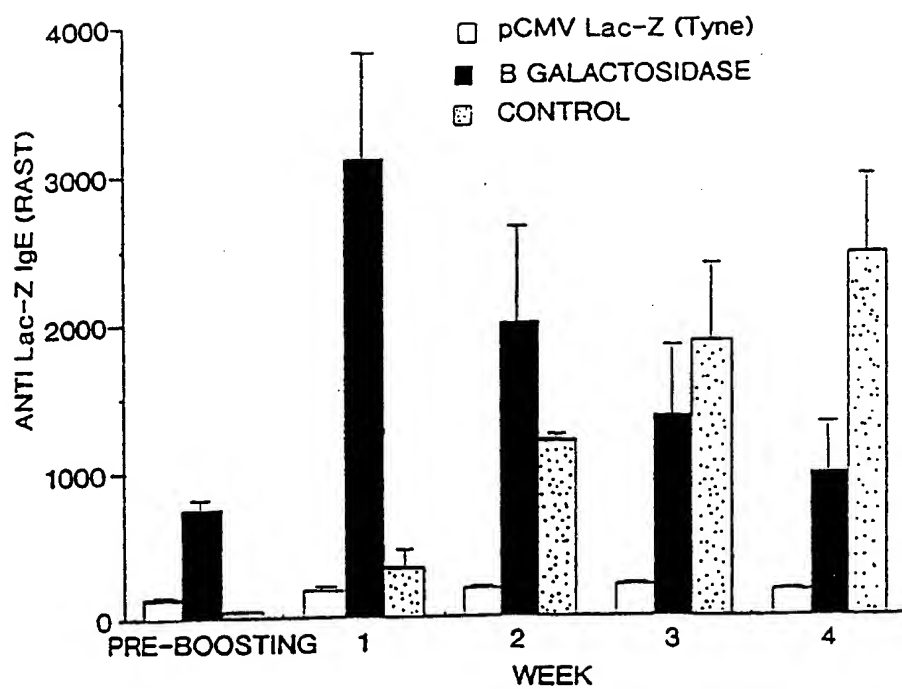


FIG. 14

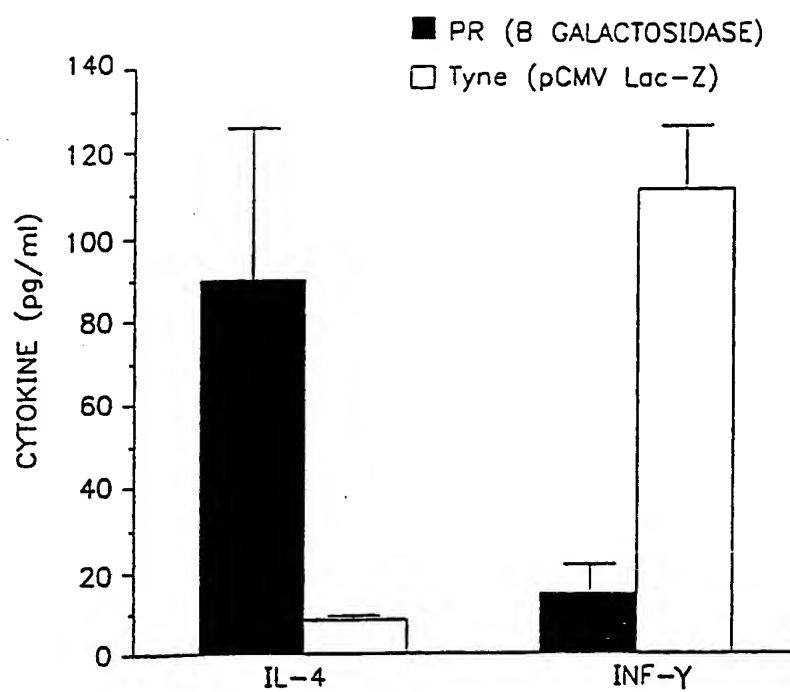


FIG. 15

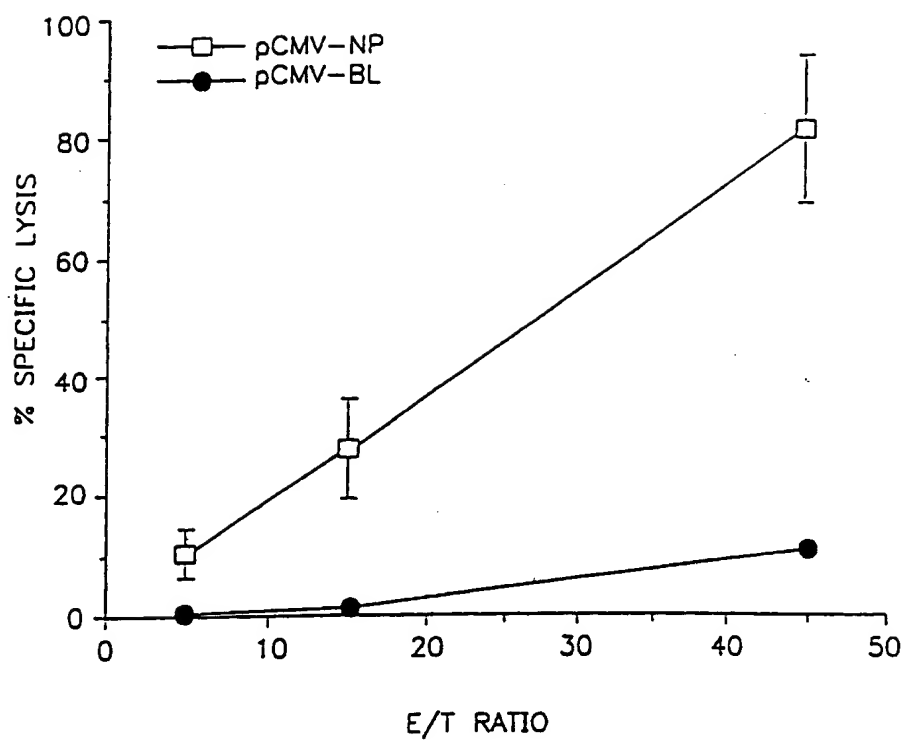


FIG. 16

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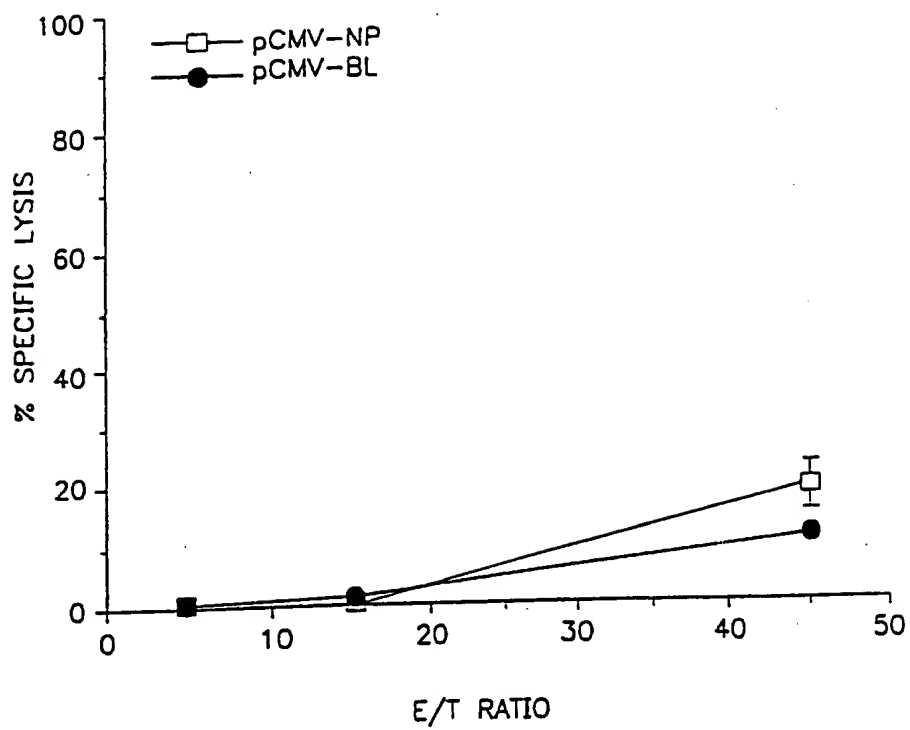


FIG. 17

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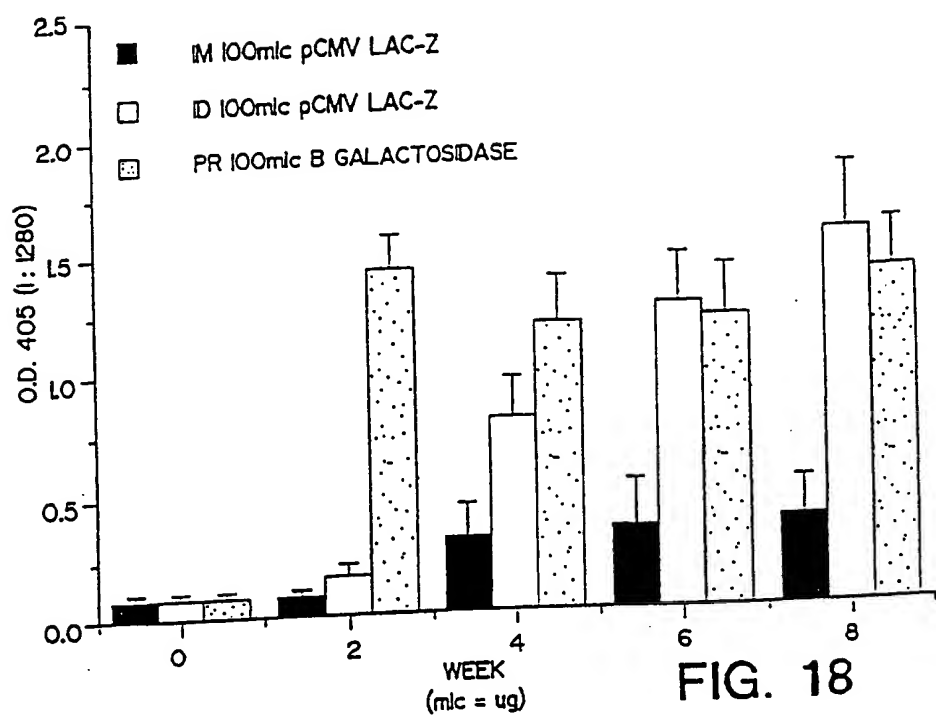


FIG. 18

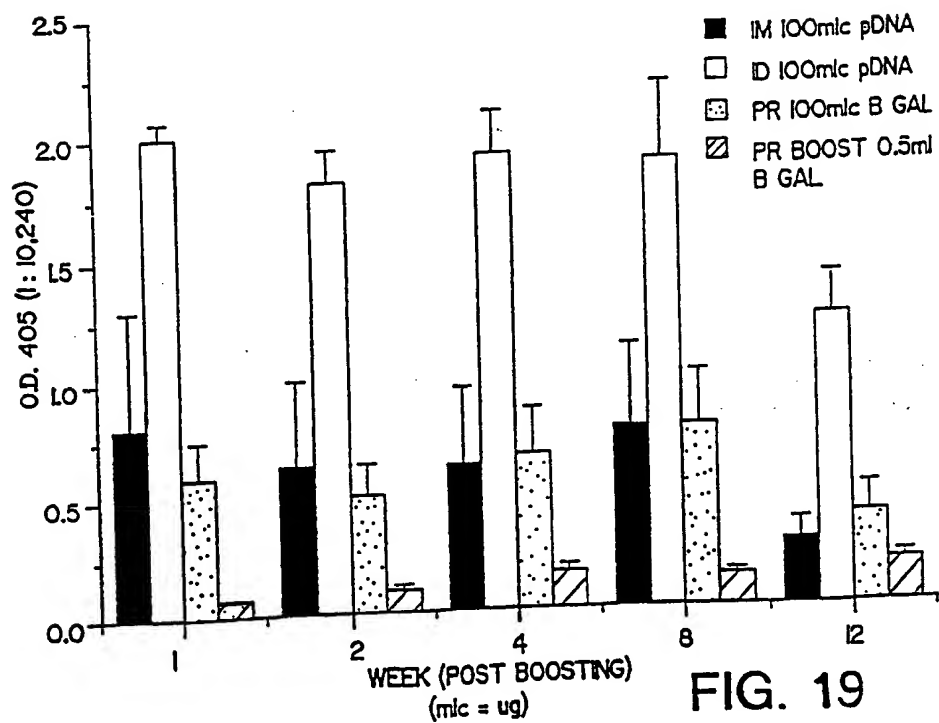


FIG. 19

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/18084

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/35 A61K48/00 A61B17/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| X | WO 95 05853 A (UNIV CALIFORNIA ;CARSON DENNIS A (US); RAZ EYAL (US); HOWELL MERED) 2 March 1995 see the whole document --- | 1-5 |
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| A | WO 96 03510 A (MERCK & CO INC ;ARMSTRONG MARCY E (US); KEYS ROBERT D (US); LEWIS) 8 February 1996 see the whole document --- | 1-5 |
| A | EP 0 620 277 A (MERCK & CO INC ;VICAL INC (US)) 19 October 1994 see the whole document --- | 1-5 |
| -/-- | | |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
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- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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- *Z* document member of the same patent family

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| Date of the actual completion of the international search 27 January 1998 | Date of mailing of the international search report 25. 02. 98 |
| Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016 | Authorized officer Hagenmaier, S |

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/18084

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|--|--|-----------------------|
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| A | COHEN: "NAKED DNA POINTS WAY TO VACCINES" SCIENCE, vol. 259, March 1993, pages 1691-1692, XP002053412 see the whole document --- | 1-5 |
| A | ULMER ET AL.: "HETEROLOGOUS PROTECTION AGAINST INFLUENZA BY INJECTION OF DNA ENCODING A VIRAL PROTEIN" SCIENCE, vol. 259, March 1993, pages 1745-1749, XP002053413 see the whole document ----- | 1-5 |

INTERNATIONAL SEARCH REPORT

international application No.
PCT/US 97/18084

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210

Although claims 1-5 (completely) are directed to a method of treatment practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/18084

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